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# Improving the molecular diagnosis of *Chlamydia psittaci* and *Chlamydia abortus* infection with a species-specific duplex real-time PCR

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*Chlamydia psittaci* and *Chlamydia abortus* are closely related intracellular bacteria exhibiting different tissue tropism that may cause severe but distinct infection in humans. *C. psittaci* causes psittacosis, a respiratory zoonotic infection transmitted by birds. *C. abortus* is an abortigenic agent in small ruminants, which can also colonize the human placenta and lead to foetal death and miscarriage. Infections caused by *C. psittaci* and *C. abortus* are underestimated mainly due to diagnosis difficulties resulting from their strict intracellular growth. We developed a duplex real-time PCR to detect and distinguish these two bacteria in clinical samples. The first PCR (PCR1) targeted a sequence of the 16S–23S rRNA operon allowing the detection of both *C. psittaci* and *C. abortus*. The second PCR (PCR2) targeted the coding DNA sequence CPSIT_0607 unique to *C. psittaci*. The two PCRs showed 100 % detection for $\geqslant$10 DNA copies per reaction (1000 copies ml$^{-1}$). Using a set of 120 samples, including bacterial reference strains, clinical specimens and infected cell culture material, we monitored 100 % sensitivity and 100 % specificity for the detection of *C. psittaci* and *C. abortus* for PCR1. When PCR1 was positive, PCR2 could discriminate *C. psittaci* from *C. abortus* with a positive predictive value of 100 % and a negative predictive value of 88 %. In conclusion, this new duplex PCR represents a low-cost and time-saving method with high-throughput potential, expected to improve the routine diagnosis of psittacosis and pregnancy complication in large-scale screening programs and also during outbreaks.

## INTRODUCTION

*Chlamydia psittaci* and *Chlamydia abortus* belong to the family *Chlamydiaceae* that comprises obligate intracellular bacteria. This family includes pathogenic members with specific host and tissue tropism responsible for a broad range of diseases both in humans and animals (Corsaro & Greub, 2006; Baud *et al.*, 2011; Asner *et al.*, 2014). *C. psittaci* is responsible worldwide for respiratory tract infections ranging from benign illness to severe pneumoniae with fatal outcomes. The primary reservoir of *C. psittaci* is birds, in particular parrots and parakeets (Longbottom & Coulter, 2003), but recent studies on chicken from the food industries and on feral pigeons suggest a significant prevalence of *C. psittaci* in these animals as well (Magnino *et al.*, 2009; Geigenfeind *et al.*, 2012; Yin *et al.*, 2013). Transmission between birds is primarily achieved through the inhalation of aerosols containing desiccated infectious particles originating from contaminated birds faeces. Infected birds can be asymptomatic hosts or develop respiratory tract disease, namely ornithosis (Longbottom & Coulter, 2003). The

Abbreviations: CDS, coding DNA sequence; RT, real-time.

Two supplementary tables are available with the online Supplementary Material.

**Table 1.** Oligonucleotide sequences

LNA, locked nucleic acid; MGB, minor groove binder; FAM, 6-carboxy-fluorescein; DQ, dark quencher; VIC, TaqMan VIC reporter dye; BHQ, black hole quencher.

| Oligonucleotide | Target gene | Sequence (5′→3′) | Modification/ fluorochrome | $T_m$ (°C) | Final concentration (μM) | Amplicon length (bp) | Specificity | Reference |
|---|---|---|---|---|---|---|---|---|
| RT-PCR | | | | | | | | |
| CPSI_F | 16S–23S rRNA operon | AAGGAGAGAGGCGCCCAA | | 59.7 | 0.35 | | *C. psittaci* and *C. abortus* | This study |
| CPSI_R_LNA | 16S–23S rRNA operon | CAA{C}CTAGTCAAACCGTCCTAA | LNA {} | 60 | 0.35 | 133 | *C. psittaci* and *C. abortus* | This study |
| CPSI_P_MGB | 16S–23S rRNA operon | ACTGGGATGAAGTCGTAAC | FAM, DQ | 66.6 | 0.2 | | *C. psittaci* and *C. abortus* | This study |
| CPSI_00F | CPSIT_0607 | AGCATTAGCCAGCGCTTTAGA | | 58.1 | 0.35 | 118 | *C. psittaci* | This study |
| CPSI_00R | CPSIT_0607 | TCTCTGAGCAAAAACACTGCGT | | 58.8 | 0.35 | 118 | *C. psittaci* | This study |
| CPSI_00R_C147G | CPSIT_0607 | TCTCTCAGCAAAAACACTGCGT | | 53 | 0.35 | 118 | *C. psittaci* | This study |
| CPSI_00P_MGB | CPSIT_0607 | ACAAAGACCTGGCGAGTA | VIC, DQ | 67 | 0.2 | | *C. psittaci* | This study |
| panCh16F2* | 16S rRNA | CCGCCAACACTGGGACT | | 60 | 0.1 | | *Chlamydiales* | Lienard *et al.* (2011) |
| panCh16R2* | 16S rRNA | GGAGTTAGCCGGTGCTTCTTTAC | | 60 | 0.1 | 207–215 | *Chlamydiales* | Lienard *et al.* (2011) |
| panCh16S | 16S rRNA | CTACGGGAGGCTGCAGTCGAGAATC | FAM, BHQ | 60 | 0.1 | | *Chlamydiales* | Lienard *et al.* (2011) |
| CpaOMP1-F | *ompA* | GCAACTGACACTAAGTCGGCTACA | | 57.4 | 0.9 | | *C. abortus* | Pantchev *et al.* (2009) |
| CpaOMP1-R | *ompA* | ACAAGCATGTTCAATCGATAAGAGA | | 52.8 | 0.9 | | *C. abortus* | Pantchev *et al.* (2009) |
| CpaOMP1-Sb | *ompA* | TAAATACCACGAATGGCAAGTTGGTTTAGCG | FAM, TAMRA | 60 | 0.2 | 82 | *C. abortus* | Pantchev *et al.* (2009) |
| PCR and sequencing | | | | | | | | |
| CPSI_OMP_F4 | *ompA* | GATCCTTGCGATCCTTGC | | 55.3 | | | *C. psittaci* and *C. abortus* | This study |
| CPSI_OMP_R1 | *ompA* | TGATAGCGGGACAAAAAGTTAGGA | | 60 | | | *C. psittaci* and *C. abortus* | This study |
| CPSI0607_SEQ_F1 | CPSIT_0607 | ATGATTAACACAGCTATCGGC | | 56.6 | | | *C. psittaci* | This study |
| CPSI0607_SEQ_R3 | CPSIT_0607 | ACTTGTTCCGCAGTTTGTTCCATC | | 62.3 | | 393 | *C. psittaci* | This study |

*Also used for PCR and sequencing.

transmission from birds to humans can be achieved through close interaction with infected birds or by inhalation of infected particles present in bird faeces (Haag-Wackernagel & Moch, 2004). The clinical expression of human psittacosis ranges from flu-like illness to an atypical severe pneumonia (Longbottom & Coulter, 2003). Recent studies demonstrated the ability of *C. psittaci* to infect bovines with the same tissue tropism as in humans, namely restriction to the respiratory tract and the same outcomes: respiratory tract infection (Reinhold *et al.*, 2012; Ostermann *et al.*, 2013). *C. abortus* can invade the placenta and lead to abortions or foetal death in the late stage of pregnancy, especially in ruminants. In sheep, *C. abortus*-induced disease is known as ovine enzootic abortion, which is one of the principal causes of lamb loss worldwide (Longbottom & Coulter, 2003). *C. abortus* can also infect other mammals such as goats, cattle, pigs, horses and deer with the same outcomes, and can also be transmitted to humans through contact with infected animal placentas that may contain a large quantity of infectious *C. abortus* organisms. *C. abortus* infection is generally asymptomatic in non-pregnant animals, but can colonize the placenta of pregnant mammals, where it causes destruction of the placental tissue ultimately resulting in foetal death and late-term abortion or miscarriage.

Both *C. psittaci* and *C. abortus* infections require rapid and accurate diagnosis in order to effectively manage infected animals and humans, and to limit or prevent their spread in animals given their epidemic and zoonotic potential. Conventional diagnostic methods that include *in vitro* isolation of the organism, immunohistochemistry and serology are time-consuming, and have limited sensitivity and specificity (Sachse *et al.*, 2009).

**Table 2.** Bacterial species used to test the specificity

| Species | Source or strain |
|---|---|
| *Escherichia coli* | ATCC 25912 |
| *Klebsiella pneumoniae* | ATCC BAA1706 |
| *Serratia marcescens* | ATCC 8100 |
| *Pseudomonas aeruginosa* | ATCC 27853 |
| *Haemophilus influenzae* | ATCC 49247 |
| *Haemophilus paraphrophilus* | ATCC 49917 |
| *Staphylococcus aureus* | ATCC 29213 |
| *Staphylococcus epidermidis* | ATCC 14990 |
| *Streptococcus pneumoniae* | ATCC 49619 |
| *Streptococcus pyogenes* | ATCC 19615 |
| *Streptococcus agalactiae* | ATCC 12386 |
| *Streptococcus mitis* | ATCC 6249 |
| *Enterococcus faecalis* | ATCC 29212 |
| *Clostridium sporogenes* | ATCC 19404 |
| *Lactobacillus* spp.* | Clinical specimen |
| *Bifidobacterium longum** | Clinical specimen |
| *Candida albicans* | ATCC 90028 |

*Identification using matrix-assisted laser desorption ionization time-of-flight MS.

We sought to improve, to accelerate and to simplify the diagnosis of *C. psittaci* and *C. abortus* infection by using real-time (RT)-PCR, which is a fast, sensitive and specific diagnostic method, in particular for intracellular pathogens. Despite their distinct host and tissue tropism, *C. psittaci* and *C. abortus* are phylogenetically so closely related that it is difficult to design a species-specific PCR targeting housekeeping genes, such as the 16S rRNA gene (Pannekoek *et al.*, 2010; Lienard *et al.*, 2011). Thus, the aim of this study was to develop a new RT-PCR-based method that could reliably detect and differentiate these two pathogens in clinical samples that could be used in routine diagnostic laboratories.

## METHODS

**RT-PCR design.** Taking advantage of the increasing number of available sequenced genomes of the family *Chlamydiaceae*, we designed a duplex RT-PCR to detect and to distinguish *C. psittaci* and *C. abortus* in clinical samples. The first RT-PCR (PCR1) targeted a region of the 16S–23S rRNA operon. This PCR could detect both *C. psittaci* and *C. abortus*. The second RT-PCR (PCR2) targeted a recently identified coding DNA sequence (CDS) present only in *C. psittaci* (Voigt *et al.*, 2012). All the primers and probes used for this study are presented in Table 1. Primers and probes were designed using Geneious 7.1.7 and Primer3Plus software. For PCR1, we performed an alignment of the 16S rRNA gene sequences available in GenBank, and selected primers specific to *C. psittaci* and *C. abortus* (Table 1). For PCR2, specific to *C. psittaci*, we used the CDS CPSIT_0607 (GenBank accession number NC_015470) predicted to encode a protein of yet undetermined function and absent from the genomes of all other *Chlamydia*, including *C. abortus*. Samples positive for both PCR1 and PCR2 were considered as positive for *C. psittaci*. When PCR1 was positive and PCR2 was negative, genital samples or samples comprising abortion products were considered positive for *C. abortus*. Considering that some strains of *C. psittaci* may not have the CDS CPSIT_0607, respiratory samples positive for PCR1 and negative for PCR2 were considered positive for *C. psittaci*.

DNA from bacterial strains or clinical samples was extracted according to the manufacturer's instructions with an LC automated system (Roche) and a MagNA Pure LC DNA isolation kit I (Roche), and resuspended into 100 µl kit elution buffer.

The RT-PCRs were performed with an ABI Prism 7900 Sequence Detection System (Applied Biosystems). The reactions were achieved in a final volume of 20 µl with 5 µl DNA sample for 45 cycles, using primers and probes at the concentrations indicated in Table 1 and TaqMan Universal Master Mix (Applied Biosystems). Cycling conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Plasmids containing the target amplicons of the RT-PCR were obtained from RD-Biotech. These plasmids were used as positive controls, and to determine the sensitivity limits and the reproducibility of the PCRs.

For each RT-PCR, a standard curve was generated by serial dilutions ranging from 5000 to 0.1 plasmid copies. This allowed the quantification of positive samples in copies per PCR, i.e. in copies/5 µl DNA.

**Sensitivity, specificity and reproducibility of the duplex PCR.** The analytic performances were assessed individually for each PCR using the corresponding positive control plasmids. The analytic sensitivity was determined with the positive control plasmids diluted from 50 to 0.01 DNA copies per reaction. We also determined that the duplexing of the PCR did not affect the analytic sensitivity of the two

**Table 3.** Bacterial DNA used to validate the duplex PCR

| Host | Species | Genotype | *C. psittaci/C. abortus* duplex PCR result | | Interpretation* |
|---|---|---|---|---|---|
| | | | PCR *C. psittaci/ C. abortus* | PCR *C. psittaci*-specific | |
| Bird | *C. psittaci* 10298 | B | + | + | *C. psittaci* |
| Bird | *C. psittaci* 10525 | B | + | + | *C. psittaci* |
| Bird | *C. psittaci* 10/282 | No typing | + | + | *C. psittaci* |
| Bird | *C. psittaci* 10/423 | B | + | + | *C. psittaci* |
| Bird | *C. psittaci* 90/1051 | A | + | + | *C. psittaci* |
| Bird | *C. psittaci* 89/1291 | A | + | + | *C. psittaci* |
| Bird | *C. psittaci* 91/0154 | A | + | + | *C. psittaci* |
| Bird | *C. psittaci* 99_3005 | A | + | + | *C. psittaci* |
| Bird | *C. psittaci* 06/020 | No typing | + | + | *C. psittaci* |
| Bird | *C. psittaci* 06/052 | No typing | + | + | *C. psittaci* |
| Bird | *C. psittaci* 2000/332 | A | + | + | *C. psittaci* |
| Bird | *C. psittaci* 91/0237 | A | + | + | *C. psittaci* |
| Bird | *C. psittaci* 96_3218 | B | + | + | *C. psittaci* |
| Bird | *C. psittaci* 98_6098 | B | + | + | *C. psittaci* |
| Bird | *C. psittaci* 96_1867_30 | B | + | + | *C. psittaci* |
| Bird | *C. psittaci* GEN_C | C | + | – | *C. psittaci* |
| Bird | *C. psittaci* GEN_D | D | + | + | *C. psittaci* |
| Bird | *C. psittaci* GEN_E | E | + | + | *C. psittaci* |
| Bird | *C. psittaci* GEN_F | F | + | + | *C. psittaci* |
| Bird | *C. psittaci* GEN_WC | WC | + | + | *C. psittaci* |
| Bird | *C. psittaci* GEN_M56 | M56 | + | – | *C. psittaci* |
| Bird | *C. psittaci* 18_290800 | E/B | + | + | *C. psittaci* |
| Bird | *C. psittaci* 3_20901 | E/B | + | – | *C. psittaci* |
| Bird | *C. psittaci* 4_20901 | E/B | + | – | *C. psittaci* |
| Bird | *C. psittaci* 5_20901 | E/B | + | – | *C. psittaci* |
| Bird | *C. psittaci* 2/290300 | E/B | + | – | *C. psittaci* |
| Ovine | *C. abortus* S263 | | + | – | *C. abortus* |
| Ovine | *C. abortus* 094_POS, Greek 'variant strain', ovine | | + | – | *C. abortus* |
| Ovine | *C. abortus* 095_A22, ovine placenta | | + | – | *C. abortus* |
| Ovine | *C. abortus* 096_S82/3, ovine placenta | | + | – | *C. abortus* |
| Ovine | *C. abortus* 097_09/04, ovine placenta | | + | – | *C. abortus* |
| Ovine | *C. abortus* 097_09/02, ovine placenta | | + | – | *C. abortus* |
| Ovine | *C. abortus* 099_11/01, ovine placenta | | + | – | *C. abortus* |
| Ovine | *C. abortus* 100_S135, ovine placenta | | + | – | *C. abortus* |
| Ovine | *C. abortus* 101_S26/3, Moredun reference strain (sequenced), ovine placenta | | + | – | *C. abortus* |
| Ovine | *C. abortus* 102_91/7, ovine placenta | | + | – | *C. abortus* |
| Ovine | *C. pecorum* 093_P787, arthritogenic strain, ovine synovial fluid | | + | – | *C. abortus*† |
| Ovine | *C. pecorum* 103_84/521F, ovine faecal strain | | + | – | *C. abortus*† |
| Bird | *C. pecorum* LW623 | | – | – | Negative |
| Bird | *C. pecorum* 66P130 | | – | – | Negative |
| Bird | *C. pecorum* 710S | | – | – | Negative |
| Bird | *C. pecorum* L71 | | – | – | Negative |
| | *C. pneumoniae* VR1310 | | – | – | Negative |
| | *Waddlia chondrophila* | | – | – | Negative |
| | *Parachlamydia acanthamoebae* | | – | – | Negative |
| | *Estrella lausannensis* | | – | – | Negative |
| | *Criblamydia sequanensis* | | – | – | Negative |
| | *Protochlamydia naegleriophila* strain Knic | | – | – | Negative |
| | *Simkania negevensis* | | – | – | Negative |

*Based on our actual knowledge on the host and tissue tropism of *C. psittaci* and *C. abortus*.

†The presence of *C. abortus* DNA was confirmed by the pan-*Chlamydiales* PCR followed by sequencing (Lienard *et al.*, 2011) and by the *C. abortus*-specific RT-PCR (Pantchev *et al.*, 2009).

PCRs. The intra- and inter-run reproducibility was assessed in duplicates in five independent runs with dilutions of the plasmids corresponding to 5000, 2000, 1000, 500, 200, 100, 50, 20, 10, 5, 2, 1, 0.5, 0.2 and 0.1 DNA copies per reactions.

For all subsequent steps of the development, the PCRs were always tested as a duplex PCR. The sensitivity and specificity were tested using DNA from bacterial strains (Table 2), from *Chlamydiales* species (Table 3) , and from human (Table 4) and veterinary (Table 5) clinical samples.

**Bacterial strains and samples.** To validate our new duplex PCR, we used a total of 120 samples, including 17 bacterial reference strains (Table 2), and 53 DNA samples obtained from cell culture infected with *C. psittaci* (at least one sample for each of all nine genotypes) and *C. abortus*. In addition, we also used bacteria phylogenetically related to these two organisms (Table 3), 22 human clinical samples matching the tissue tropism of *C. abortus* and *C. psittaci*, and 33 veterinary samples.

**Gold standard and discrepant results investigation.** For the investigation of discrepant results, we designed the primers CPSI_OMP_F4 and CPSI_OMP_R1 for the amplification and the sequencing of the *ompA* gene of *C. psittaci* and *C. abortus* (Table 1). We also used the *Chlamydiales*-specific RT-PCR developed by Lienard *et al.* (2011) that targeted the 16S rRNA gene. The PCR product of the *Chlamydiales*-specific RT-PCR could be further sequenced using the primers panCh16F2 and panCh16R2. The *C. abortus* specific RT-PCR targeting the *ompA* gene was used to detect *C. abortus* (Pantchev *et al.*, 2009). The binding region of the primer CPSI_00F, CPSI_00R and the probe CPSI_00P_MGB on the CDS CPSIT_0607 was amplified using the primers CPSI0607-SEQ-F1 and CPSI0607-SEQ-R3. Elongation was performed using the primers CPSI_00F and the primer CPSI0607-SEQ-R3 (Table 1).

**Case studies.** To further validate the new duplex RT-PCR, we analysed a number of local cases. Each positive sample was analysed both with the *Chlamydiales*-specific RT-PCR as described by Lienard *et al.* (2011) and with the new duplex RT-PCR.

## RESULTS

### Target gene, primers and probes

Despite different host specificity and tissue tropism, *C. psittaci* and *C. abortus* are phylogenetically closely related bacteria. This prevents the differentiation of these two organisms by a single RT-PCR targeting a housekeeping gene. Thus, we decided to develop a duplex PCR consisting of (i) a PCR (PCR1) targeting a housekeeping gene that allowed the detection of both organisms, but not their differentiation, and (ii) a second PCR (PCR2) targeting a gene that allowed their specific differentiation. The first PCR targeted the intergenic spacer of the 16S–23S rRNA operon (Table 1), whilst the second PCR targeted the *C. psittaci* CDS CPSIT_0607. A comparative genomic study showed that the CDS CPSIT_0607 is unique to *C. psittaci* (Voigt *et al.*, 2012).

**Table 4.** Clinical specimen used to validate the duplex PCR

| Sample (*n*) | Gold standard identification | *C. psittaci*/*C. abortus* duplex PCR result | | Interpretation of the duplex PCR |
|---|---|---|---|---|
| | | PCR *C. psittaci*/*C. abortus* | PCR *C. psittaci*-specific | |
| Uterus and urethral smear | *C. abortus** | + | – | *C. abortus* |
| Uteral swab | *C. trachomatis* | – | – | Negative |
| Mouth swab (3) | Oropharyngeal commensal flora | – | – | Negative |
| Nasopharyngeal secretions (2) | *C. pneumoniae* | – | – | Negative |
| Nasopharyngeal secretions (2) | *Mycoplasma pneumoniae*† | – | – | Negative |
| Bronchoalveolar lavage (2) | *Legionella pneumophila*† | – | – | Negative |
| Nose swab | *Bordetella pertussis* | – | – | Negative |
| Nasopharyngeal secretions (2) | *Bordetella pertussis* | – | – | Negative |
| Expectoration | *Mycobacterium tuberculosis* | – | – | Negative |
| Bronchoaspiration | *Mycobacterium tuberculosis* | – | – | Negative |
| Bronchoaspiration | *Aspergillus fumigatus* | – | – | Negative |
| Bronchoalveolar lavage | *Aspergillus fumigatus* | – | – | Negative |
| Bronchoalveolar lavage | *Pneumocystis jirovecii* | – | – | Negative |
| Bronchoaspiration | *Pneumocystis jirovecii* | – | – | Negative |
| Drain fluid from aortic valve | *Coxiella burnetii* | – | – | Negative |
| Protetic fragment | *Coxiella burnetii* | – | – | Negative |

*Pan-*Chlamydiales* PCR followed by sequencing (Lienard *et al.*, 2011).
†*M. pneumoniae/L. pneumophila* duplex RT-PCR (Welti *et al.*, 2003).

**Table 5.** Veterinary specimens used for validation of the duplex PCR

| Sample type | Gold standard identification | *C. psittaci*/*C. abortus* duplex PCR result | | *C. psittaci*/*C. abortus* duplex PCR interpretation |
|---|---|---|---|---|
| | | PCR *C. psittaci*/ *C. abortus* | PCR *C. psittaci*-specific | |
| Bird spleen | *C. psittaci** | + | + | *C. psittaci* |
| Fragment of sheep placenta | *C. abortus** | + | − | *C. abortus* |
| Fragment of sheep placenta | *C. abortus** | + | − | *C. abortus* |
| Biopsies of sheep placenta† | | | | |
| Biopsy 307A | *C. abortus* | + | − | *C. abortus* |
| Biopsy 439P | *C. abortus* | + | − | *C. abortus* |
| Biopsy 452A | *C. abortus* | + | − | *C. abortus* |
| Biopsy 511P | *C. abortus* | + | − | *C. abortus* |
| Biopsy 512P | *C. abortus* | + | − | *C. abortus* |
| Biopsy 545A | *C. abortus* | + | − | *C. abortus* |
| Biopsy 745P | *C. abortus* | + | − | *C. abortus* |
| Biopsy 1725N | *C. abortus* | + | − | *C. abortus* |
| Biopsy 2571N | *C. abortus* | + | − | *C. abortus* |
| Biopsy 451P | *C. abortus* | + | − | *C. abortus* |
| Biopsy 828P | *C. abortus* | + | − | *C. abortus* |
| Biopsy 851P | *C. abortus* | + | − | *C. abortus* |
| Biopsy 1504N | *C. abortus* | + | − | *C. abortus* |
| Biopsy 1535N | *C. abortus* | + | − | *C. abortus* |
| Biopsy 1683N | *C. abortus* | + | − | *C. abortus* |
| Biopsy 2542N | *C. abortus* | + | − | *C. abortus* |
| Biopsy 254B | *C. abortus* | + | − | *C. abortus* |
| Biopsy 314A | *C. abortus* | + | − | *C. abortus* |
| Biopsy 717A | *C. abortus* | + | − | *C. abortus* |
| Biopsy 1019P | *C. abortus* | + | − | *C. abortus* |
| Biopsy 1568N | *C. abortus* | + | − | *C. abortus* |
| Biopsy 2537N | *C. abortus* | + | − | *C. abortus* |
| Biopsy 637P | *C. abortus* | + | − | *C. abortus* |
| Biopsy 758A | *C. abortus* | + | − | *C. abortus* |
| Biopsy 1509N | *C. abortus* | + | − | *C. abortus* |
| Biopsy 1088D | Negative | − | − | Negative |
| Biopsy 1086D | Negative | + | − | *C. abortus*‡ |
| Biopsy 1092D | Negative | + | − | *C. abortus*‡ |
| Biopsy 723P | Negative | + | − | *C. abortus*§ |
| Biopsy 572P | Negative | + | − | *C. abortus*§ |

*Pan-*Chlamydiales* PCR followed by sequencing (Lienard *et al.*, 2011).
†Longbottom *et al.* (2013).
‡*C. abortus* DNA also detected with the pan-*Chlamydiales* PCR and with the *C. abortus*-specific RT-PCR *ompA*.
§Infected animal, *C. abortus* DNA also detected with the pan-*Chlamydiales* PCR and with the *C. abortus*-specific RT-PCR *ompA*.

## Analytical sensitivity and reproducibility of the two RT-PCRs

The sensitivity of the two RT-PCRs was evaluated with five replicates of different plasmid control concentrations (50, 20, 10, 5, 2, 1, 0.5, 0.2 and 0.1 copies per reaction). PCR1 showed 100 % detection for $\geq$10 DNA copies per reaction, 90 % for 5 and 2 copies per reaction, and 60 % for 1 copy per reaction (Fig. 1a). PCR2 showed 100 % detection for $\geq$5 DNA copies per reaction, 80 % for 2 copies per reactions and 50 % for 1 copy per reaction (Fig. 1b). The intra- and inter-run reproducibility of PCR1 and PCR2 was assessed on five independent runs in duplicates using serial dilutions of the positive control plasmid ranging from 5000 to 0.1 DNA copies $ml^{-1}$ (Fig. 2). When plotting the cycle threshold ($C_t$) values of duplicates of the same amplification, we calculated a $R^2$ of 0.96 for PCR1 and 0.97 for PCR2, which is excellent (Fig. 2a, b). To test the inter-run reproducibility, we plotted the 10-copy and 100-copy positive control of 10 successive runs, which revealed that only one 10-copy positive control exhibited a $C_t$ value that was $>2$SD of the mean (Fig. 2c).

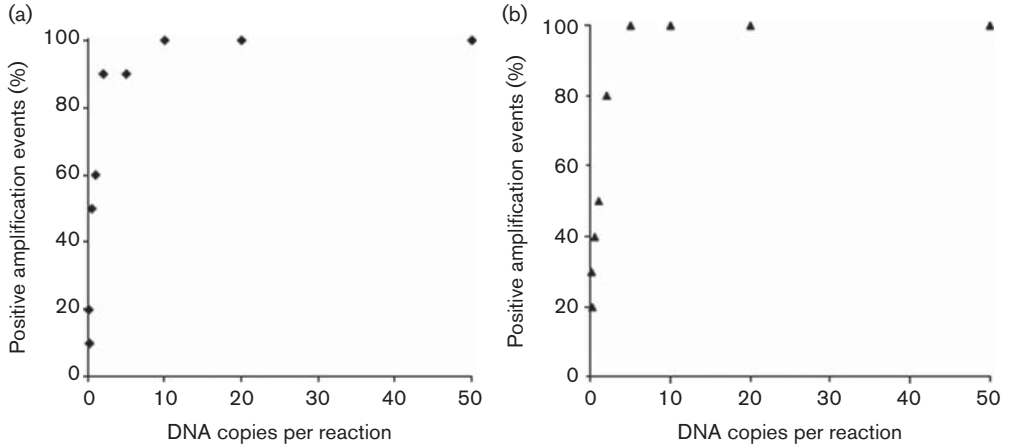


**Fig. 1.** Analytic sensitivity of the two RT-PCRs: (a) PCR1 and (b) PCR2.The analytic sensitivity was evaluated with five replicates of different plasmid control concentrations (50, 20, 10, 5, 2, 1, 0.5, 0.2 and 0.1 copies per reaction) for both PCRSs.

## Performance of the duplex RT-PCR

The overall performance of the duplex PCR was assessed with a total of 120 samples. No amplification was observed with 17 bacterial strains (15 ATCC and two clinical isolates), including bacteria sharing the same tissue tropism as *C. psittaci* or *C. abortus* (Table 2).

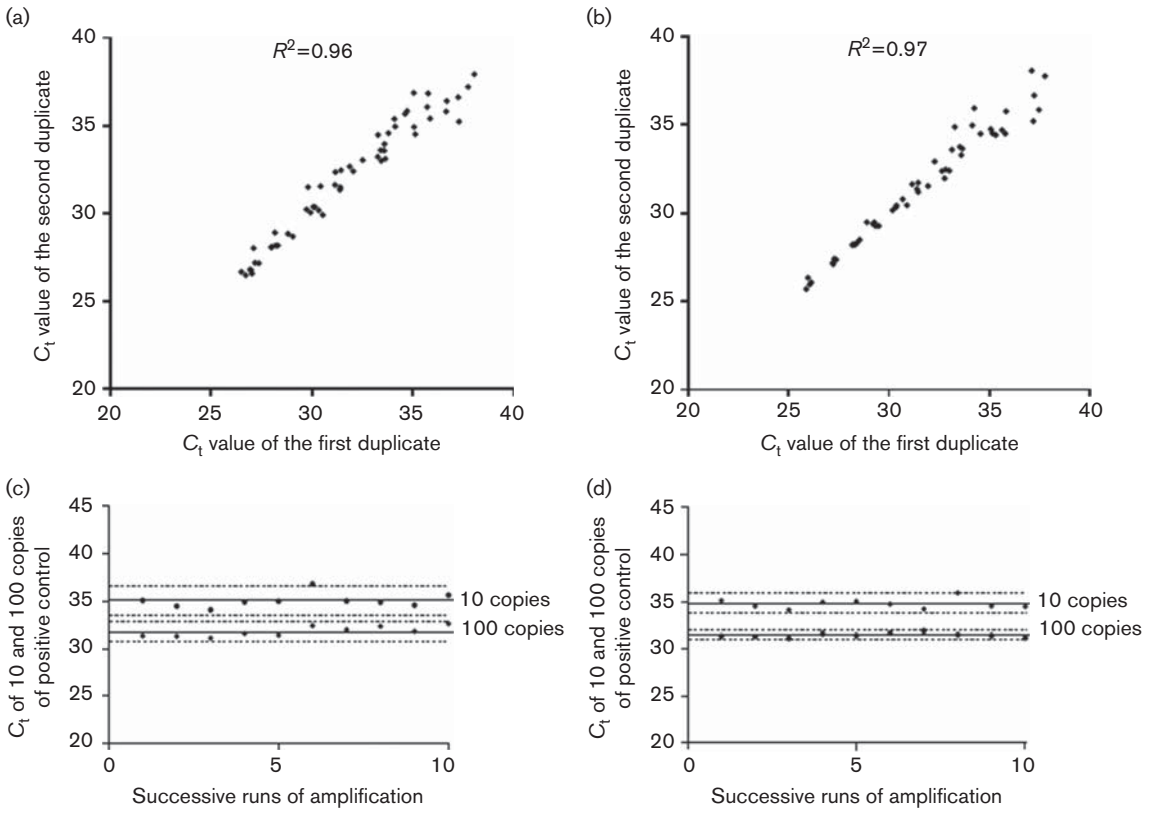


**Fig. 2.** Reproducibility of the two RT-PCRs. (a, b) Intra-run variability of PCR1 (a) and PCR2 (b) between duplicates of the control plasmid. (c, d) Inter-run variability using 10 and 100 plasmid copies, as obtained during 10 successive runs for PCR1 (c) and PCR2 (d); the solid black lines show the mean $C_t$ values and the dashed lines each side of the means indicate 2SD.

Fifty-three samples used to test the specificity of the PCRs were DNA samples (obtained from infected cell culture material) from *C. psittaci* and *C. abortus*, and phylogenetically related bacteria, namely *Chlamydia pecorum* and *Chlamydia pneumoniae*, *Waddlia chondrophila*, *Parachlamydia acanthamoebae*, *Estrella lausannensis*, *Criblamydia sequanensis*, *Protochlamydia naegleriophila* strain Knic, and *Simkania negevensis* (Table 3). The results of the duplex PCR were correct for 45 of the 53 samples, with eight results being discordant. Six of them corresponded to DNA samples obtained from cell cultures infected with *C. psittaci* genotype E/B, C and M56 that were found positive for the PCR1 and negative for the PCR2 targeting the CDS CPSIT_0607. The analysis of the genome of these three genotypes revealed that they were devoid of the homologue of the CDS CPSIT_0607 (Van Lent *et al.*, 2012). The two other discordant results corresponded to samples thought to contain *C. pecorum* DNA. These samples were positive for PCR1 and negative for PCR2 with our duplex PCR, and thus interpreted as positive for *C. abortus*. These two samples were further analysed both with the *Chlamydiales*-specific PCR targeting the 16S rRNA sequence (Lienard *et al.*, 2011) and a *C. abortus*-specific PCR targeting the *ompA* gene (Pantchev *et al.*, 2009). Both PCRs were positive for *C. abortus*, thereby confirming the presence of *C. abortus* DNA and validating our new duplex PCR.

The samples tested with the duplex PCR also included 22 human clinical samples, whose selection was biased towards genital and respiratory tract samples, matching the tissue tropism of *C. abortus* and *C. psittaci*, respectively. One sample was a uterus and urethral smear that was positive for *C. abortus* (corresponding to case study 1, see below). The other samples were all negative for *C. psittaci* and *C. abortus*. We obtained 100 % concordance between our duplex PCR and the indicated gold standard (Table 4). We next tested the duplex PCR on 33 veterinary samples. The sample corresponding to a bird spleen positive for *C. psittaci* (case study 2) was also identified as *C. psittaci* with our new duplex PCR. The 27 ovine placental samples positive for *C. abortus* (Longbottom *et al.*, 2013) were all correctly identified by our duplex PCR. No amplification was obtained with sample 1088D that was negative for both *C. abortus* and *C. psittaci* according to our collaborator gold standard (Longbottom *et al.*, 2013). In contrast, we obtained amplification with PCR1, but not with PCR2, revealing the presence of *C. abortus* DNA in samples 1086D, 1092D, 723P and 572P. These samples were found positive for *C. abortus* with the pan-*Chlamydiales* PCR and the *C. abortus*-specific PCR targeting the *ompA* gene (Pantchev *et al.*, 2009) (Table 5).

On the basis of these 120 samples, we determined 100 % sensitivity and 100 % specificity for our new *C. psittaci* and *C. abortus* duplex PCR (Table 6, PCR1). Moreover, when PCR1 was positive, PCR2 could differentiate *C. psittaci* from *C. abortus* with a positive predictive value of 100 % and a negative predictive value of 88 % (Table 6, PCR2).

## Case studies

The new duplex PCR was introduced into our diagnostic laboratory for a retrospective and prospective case study analysis. Using our new duplex RT-PCR, we could confirm one case of ornithosis (Senn & Greub, 2008) and one case of *C. abortus* infection. We could also diagnose two cases of psittacosis. All these cases are summarized in Table 7 and in Table S1 (available in the online Supplementary Material).

**Table 6.** Performance of the duplex PCR

The duplex PCR was tested on 120 samples. The performance of PCR1 that could detect *C. psittaci* and *C. abortus* was calculated on all 120 samples. The performance of PCR2 specific for *C. psittaci* was calculated on the 69 samples positive for PCR1.

| | | ***C. psittaci* or *C. abortus*** | **Other than *C. psittaci* or *C. abortus*** | | |
|---|---|---|---|---|---|
| PCR1 | Correct for *C. psittaci* or *C. abortus* | 71 | 0 | 100 % | Positive predictive value |
| | Negative | 0 | 49 | 100 % | Negative predictive value |
| | | 100 % | 100 % | 120 | |
| | | Sensitivity | Specificity | Total no. of samples | |
| | | ***C. psittaci*** | ***C. abortus*** | | |
| PCR2 | Positive | 21 | 0 | 100 % | Positive predictive value |
| | Negative | 6 | 42 | 88 % | Negative predictive value |
| | | 78 % | 100 % | 69 | |
| | | Sensitivity | Specificity | Total no. of samples | |

### Optimization of PCR1

The CDS CPSIT_0607 was chosen as the target for PCR2 on the basis of a comparison of the genome of *C. psittaci* 6BC with the genomes of other members of the *Chlamydiaceae* (Voigt *et al.*, 2012). Our *in silico* analysis first identified five homologues of the CDS CPSIT_0607 in *C. psittaci* C19/98, *C. psittaci* 01DC11, *C. psittaci* 02DC15, *C. psittaci* 08DC60 (Schöfl *et al.*, 2011) and *C. psittaci* RD1 (Seth-Smith *et al.*, 2011). The specific primers and the probe for PCR2 were designed using an alignment of these six sequences. However, the sequencing of the target of PCR2 in additional *C. psittaci* positive samples revealed a polymorphism (C147G) in the binding region of the reverse primer CPSI_00R. This mutation was predicted to induce a 5.8 °C decrease in the melting temperature ($T_m$) of the oligonucleotide CPSI_00R, but the $C_t$ of PCR1 and PCR2 were similar in *C. psittaci*-positive samples with the mutation C147G (Table S2). Thus, the mutation C147G did not impact the sensitivity of our duplex PCR. Nevertheless, we decided to update our RT-PCR by using a combination of two reverse primers, namely CPSI_00R and CPSI_00R_C147G. We tested the duplex PCR containing these new primers on five *C. psittaci* DNA samples containing the C147G mutation. The PCR containing the reverse primer CPSI_00R_147C/G was as efficient at amplifying the target *C. psittaci* sequence as the PCR containing the original primer CPSI_00R (Table S2).

## DISCUSSION

We have developed a new duplex RT-PCR that is able to rapidly detect and distinguish *C. psittaci* and *C. abortus* in clinical samples. The first PCR, which is based on the 16S rRNA gene, is able to identify *C. psittaci* and *C. abortus* with 100 % sensitivity and specificity with a sensitivity limit $<10$ DNA copies per reaction. This PCR is suitable for the screening of *C. psittaci* and *C. abortus* because of the 100 % sensitivity for the two species. The second PCR that targets the CDS CPSIT_0607 found in the genome of *C. psittaci* isolate 6BC and absent from all known *C. abortus* genomes allows differentiating these two pathogens. The specific PCRs published to date for the detection of *C. psittaci* and *C. abortus* target highly polymorphic genes, such as the *ompA* gene encoding the major outer membrane protein (Pantchev *et al.*, 2009). So far, none of the published *C. psittaci*/*C. abortus* RT-PCRs have fulfilled the characteristics needed to be implemented in our automated molecular diagnosis platform based on TaqMan technology and using standardized PCR in terms of length of the amplicon, $T_m$ of the primers and probes, and elongation temperature. This standardization allows us to run all the different RT-PCRs in batches in 384 multi-well plates. Thus, currently, we are able to detect $>65$ different pathogens using similar homemade TaqMan PCRs, including several intracellular bacteria such as *C. trachomatis* (Jaton *et al.*, 2006), *C. pneumoniae* (Welti *et al.*, 2003) and *Coxiella burnetii* (Jaton *et al.*, 2013). Branley *et al.* (2008) have developed a PCR targeting the 16S rRNA gene, but using SYBR Green technology, that presented a $T_m$ lower than the target $T_m$ of our platform (60 °C) (Branley *et al.*, 2008). Most of the species-specific RT-PCRs target outer membrane proteins with a high risk of loss of sensitivity due to single nucleotide polymorphisms (Creelan & McCullough, 2000; Heddema *et al.*, 2006a, 2015; Pantchev *et al.*, 2009). The *incA* gene encoding the inclusion membrane protein A has been used to design a *C. psittaci*-specific PCR, but published primers did not reach the $T_m$ of our automated platform (Ménard *et al.*, 2006). We recently developed a pan-*Chlamydiales* RT-PCR targeting the 16S rRNA gene able to detect the presence of *Chlamydiales* species in clinical or environmental samples with a high sensitivity (5 DNA copies per reaction) and high specificity. Nevertheless, when positive, a further sequencing of the PCR amplicons is required to identify the bacteria at the species level (Lienard *et al.*, 2011). Thus, our new duplex PCR is a straightforward method for the rapid diagnostic of *C. psittaci* and *C. abortus* infections on a molecular diagnosis platform using TaqMan technology.

During the validation of our new duplex PCR, we found that the available genomes of *C. psittaci* genotypes A, B, D, E, F and WC contain orthologues of the CDS CPSIT_0607 (Van Lent et al.,, 2012). In contrast, the available genomes of *C. psittaci* genotype E/B (identified in ducks, cattle and muskrats), genotype C (isolated from ducks and geese) and genotype M56 (isolated in muskrats) are devoid of this CDS (Geens *et al.*, 2005; Van Lent *et al.*, 2012). The positive predictive value for the identification of *C. psittaci* is 100 % as *C. abortus* strains also lack this CDS. The negative predictive value that we report (88 %) might be different in other regions as it depends on the prevalence of the genotype lacking the target gene. The analysis of more *C. psittaci* genomes will refine the distribution of strains lacking this gene. Thus, samples positive for both PCR1 and PCR2 can accurately be identified as containing *C. psittaci* DNA. Conversely, genital samples or samples consisting of abortion products positive for PCR1 and negative for PCR2 were presumably identified as *C. abortus*. Respiratory samples positive for PCR1 and negative for PCR2 were considered as putatively positive for *C. psittaci*, especially in the context of bird exposure.

We also performed an *in silico* survey of the new *Chlamydiaceae* DNA sequences submitted together with the sequencing of the target gene of PCR2 of our duplex PCR. This allowed us to identify a polymorphism that could affect the binding of the reverse primer of this PCR. This led us to design a degenerate primer to prevent any decrease in PCR sensitivity. Our development is an example of the importance of a continuous *in silico* and *in vitro* survey of the reliability of PCR-based molecular diagnosis methods to overcome the appearance of new pathogen variants that could be missed. This is particularly true for pathogens for which the number of available sequences is limited (e.g. emerging pathogens) or for pathogens with a high rate of mutations, such as viruses.

**Table 7.** Case studies

| Case study | Sample | Interpretation of the duplex PCR *C. psittaci/C. abortus* | PCR1 result | PCR2 result | Clinical features |
|---|---|---|---|---|---|
| 1: *C. abortus* infection of a pregnant woman 2012* | Uterus and urethral smear from a pregnant woman | *C. abortus* | + | – | Pregnant woman in contact with a flock of sheep |
| | Fragment of sheep placenta from an abortive sheep | *C. abortus* | + | – | Abortion of the sheep |
| | Fragment of sheep placenta from a second abortive sheep | *C. abortus* | + | – | Abortion of the sheep |
| 2: Parrot from a public park† | Parrot spleen | *C. psittaci* | + | + | Dead parrot from a public park dissected by an employee of a veterinary clinic that subsequently developed psittacosis |
| 3: Pigeon owner 2013* | Expectoration from the pigeon owner | *C. psittaci* | + | + | Fever and haemoptysis; radiologic condensations of the basoposterior segment of the right lower lobe of the lung in a patient in contact with a pigeon loft |
| | Pigeon choanal and cloacal swab | *C. psittaci* | + | + | Death of the pigeon |
| | Dust from the pigeon loft | *C. psittaci* | + | + | – |
| 4: Worker on the roof of a building* | Endotracheal secretions | *C. psittaci* | + | + | Respiratory illness in a patient working on the roof of buildings |

*This study.
†Senn & Greub (2008).

The zoonotic incidence of *C. psittaci* and *C. abortus* is well documented, but their incidence in human diseases might be underestimated (Braukmann *et al.*, 2012; Geigenfeind *et al.*, 2012). In particular, ~50 % of pneumonia cases remain diagnosed as of unknown aetiology. *C. psittaci* should especially be considered in the differential diagnosis if history reveals an exposure to birds. The situation is similar for abortions, where nearly 50 % of cases are of unexplained aetiology (Baud *et al.*, 2008) and could sometimes be caused by *C. abortus*, particularly in rural areas or following exposure to sheep at lambing. The sensitivity and the specificity of our new duplex PCR make it a highly reliable diagnostic test to confirm or to exclude suspected *C. psittaci* or *C. abortus* infections during routine diagnosis. Moreover, the high-throughput potential together with the short turnaround time and the low cost of this RT-PCR make it a powerful tool for large-scale screening programs during outbreaks, if such situations arise. This may be very important, as the high infectivity of *C. psittaci* by the airway route confers a significant outbreak potential (Heddema *et al.*, 2006b; Gaede *et al.*, 2008; Belchior *et al.*, 2011; McGuigan *et al.*, 2012) and consideration as a potential bioterrorism agent.

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